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IMMUNE RESPONSES AGAINST MYCOBACTERIUM TUBERCULOSIS TARGETS ASSOCIATED TO LATENT AND ACTIVE TUBERCULOSIS INFECTION

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Immune responses against *Mycobacterium tuberculosis* targets associated to latent and active tuberculosis infection
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“The fear of the Lord is the beginning of wisdom,

and knowledge of the Holy One is understanding”

Proverbs 9:10

A mis queridos padres, y toda mi bella familia

To my dear parents and my beautiful family

ABSTRACT

Background: Tuberculosis is a worldwide problem particularly in developing countries. Different clinical outcomes, such as the asymptomatic phase during infection or the symptomatic stage during active disease leads to activation and expansion of cellular immune responses in order to control the infection. This process of latency and active TB infection is a highly dynamic interaction between the host and the immunogenicity of *M.tb*. The detailed antigen specific analysis of the *M.tb* host response will likely lead to a better understanding of the pathogenesis of TB in human subjects and may help to identify markers of immune protection. There is a need to develop an effective TB vaccine and biomarkers of protection. Currently, different TB vaccine candidates contain *M.tb* components that are able to induce T cell activation and subsequent IFN-γ release. For instance, TB10.4 is such a novel vaccine candidate, it is highly immunogenic and present in *M.tb* and in the BCG vaccine strain. We characterized immune recognition patterns from Honduran patients with TB with a particularly focus on TB10.4 in order to better understand the infection biology, heterologous immune responses and the role of memory T cell formation.

Aims: i) Defining and comparing *M.tb* specific immune responses in individuals with active and latent tuberculosis infection using cell based immune assays and a panel of specific *M.tb* target antigens ii) Characterization of the recognition pattern of the humoral and cellular responses towards TB10.4 (Rv0288) and iii) Studying the relationship between molecular mimicry and tuberculosis using TB10.4 as a paradigm.

Methodology: We selected a clinically well-defined population in order to perform cell-based immune assays (i.e Interferon gamma release assay), microarray peptide technology, and a bioplex assay, to gauge immune responses against a panel of recombinant or synthetic *M.tb* target antigens.

Results and discussion: **Paper I**: This work represents the first analysis of cellular immune responses directed against *M.tb* associated targets in individuals from Honduras and provides a robust concept for identification of TB targets; we were able to identify differential target recognition patterns in TB+ patients vs TB exposed health care workers. Comprehensive pattern recognition analysis using biologically relevant targets revealed that enzymes involved in *M.tb* lipid synthesis serve as targets for T cells, defined by IFN-γ and IL-17 production. **Paper II:** We mapped humoral and cellular immune responses against the TB10.4 protein in non-human primates (NHPs) as well as in TB patients from Honduras. Immune responses in NHP were very focused, in contrast toTB10.4 directed immune responses in humans. We postulated that cross recognition of closely related antigens could be one possible explanation. The report shows substantial differences in cellular recognition comparing NHP and human immune responses. This T cell responses may be sustained via cross reactive immune responses recognizing the TB10.4 target and non-*M.tb* related target antigens.

LIST OF SCIENTIFIC PAPERS

I. Differential cellular recognition pattern to *M. tuberculosis* targets defined by IFN-y and IL-17 production in blood from TB+ patients from Honduras as compared to health care workers: TB and immune responses in patients from Honduras. *BMC Infectious Diseases* 2013, **13**:125 doi:10.1186/1471-2334-13-125.

Nancy Alvarez Corrales, Raija K Ahmed, Carol A Rodriguez, Kithiganahalli N Balaji, Rebeca Rivera, Ramakrishna Sompallae, Nalini K Vudattu, Sven E Hoffner, Alimuddin Zumla, Lelany Pineda Garcia and Markus Maeurer.

II. Immune reactivity to *Mycobacterium tuberculosis* TB10.4 epitopes and molecular mimicry. Manuscript under review

Nancy Alvarez-Corrales, Lelany Pineda-Garcia, Raija Ahmed, Isabelle Magalhaes, Davide Valentini, Chaniya Leepiyasakulchai, Carol Rodriguez, Jerry Sadoff, Alimudin Zumla, Markus Maeurer

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LIST OF ABBREVIATIONS

TB	Tuberculosis
M.tb	Mycobacterium tuberculosis
LTBI	Latent tuberculosis infection
CMI	Cell mediated immunity
IL	Interleukin
IFN-γ	Interferon gamma
IL-17	Interleukin 17
Th1	T helper lymphocyte 1
Th2	T helper lymphocyte 2
DC	Dendritic cell
TCR	T cell receptor
MHC	Major histocompatibility complex
APC	Antigen presenting cell
CFP-10	Culture filtrate protein – 10
ESAT-6	Early secreted antigen
BCG	Bacille Calmette Guerin
IGRA	Interferon gamma release assay
QFT-GIT	Quantiferon TB Gold in tube
WBA	Whole blood assay
ELISA	Enzyme linked Immunosorbent assay
AFS	Acid fast staining

1 EPIDEMIOLOGY OF TUBERCULOSIS, CHAPTER 1

1.1 GLOBAL AND REGIONAL FACTS OF TUBERCULOSIS

Tuberculosis (TB) is an increasing threat worldwide contributing to morbidity and mortality among young adults, caused by *Mycobacterium tuberculosis* (*M.tb*).
Pulmonary tuberculosis is an aerosol infection and represents the most prevalent form of the disease; this contagious airborne form implies a global public health problem. 1.3 million people succumb to TB, including 320,000 individuals with HIV infections and two billion people living with Latent TB infection (LTBI). The incidence is 137 cases per 100,000 with therapy outcome that reaches 86% [1] , if *M.tb* is susceptible to antibiotic treatment.
Nowadays, the global challenge is to develop new strategies of treatment and diagnostics for preventing the multidrug resistance (MDR-TB) and extensively Drug resistance (XDR-TB) form of TB that fail to respond to first and second line of anti TB therapy. Therefore, a global plan to stop TB, attributed to prioritize 22 High Burden Countries (HBC) that represent 80% of TB cases around the world, counting 35% alone for India and China [2-3] was developed.
The region of America is one of the WHO' six regions with a TB incidence of 29/100000, a prevalence of 37/100000, showing 82% of treatment success; 41% of patients with TB tested HIV-positive. The Americas include only one HBC-Brazil and the regional plan 2006-2015 attributed Brazil 35% of the cases in the Region followed by Peru (15%)[3].
In Central America the highest rate of TB during 2012 correspond to Guatemala, followed by Honduras with an incidence of 60 and 54/100,000 respectively[4-5].
During 2012, 3,014 new and relapsed cases of tuberculosis were detected in Honduras; 2816/3014 TB patients were HIV-positive[5]. HIV remains a serious public health problem increasing the risk factor for progression to active disease. 1.8% patients with TB experience MDR-TB[5], they fail to limit the active form of the disease and fail to respond to first and second line drugs.
The ability to contain TB latency worldwide can be maintained during life time unless host immunity or environmental factors compromise such a status. HIV co-infection, malnutrition, crowds, aging, stress, type 2 diabetes mellitus, use of immunosuppressive agents and genetic factors may contribute to progression of TB infection. Yet, other factors from the pathogen, may also play a role: latency reflects the transition from replicating to no replicating dormant bacilli, this transition is influenced by diverse factors, including oxygen deprivation and nitric oxide formation.

To control TB expansion, nations have developed political commitment and strategies promoting actions for prevention, developing diagnostics, novel drug therapy and monitoring TB instruments to gauge the TB epidemic.

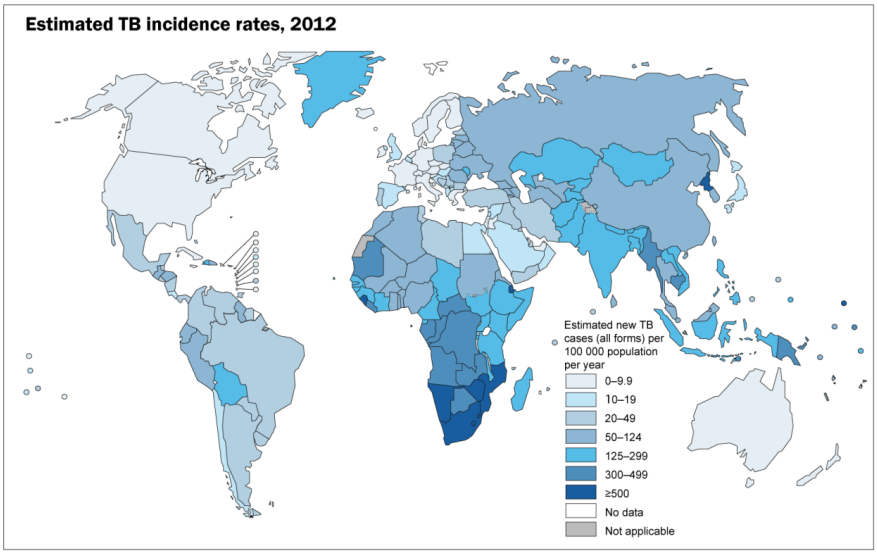
In Honduras, the health care system is comprised by the Ministry of Health, Social Security Institute (IHSS) and the Private Sector; all of them providing assistance in tuberculosis services. Public service includes 6 National Hospitals; at the intermediate level 20 Health Regions, 6 Regional Hospitals, 16 Areas Hospitals; and the local level 252 CESAMO, 1058 CESAR and 4 CLIPER (smaller and peripheral health centers). Among primary tuberculosis care institutions is the National TB Control Program (NTP) [6] which was established in 1957 with strategies to control TB in Honduras and to develop pilot activities using Directly Observed Therapy, Short-Course (DOTS) in 1998; by 2003, the NTP reported 100 percent DOTS coverage[7] and achieved a treatment success rate of 86 percent, a marked improvement from 75 percent in 1998.

Box 1. Honduras General and Health Facts

Capital City	*Tegucigalpa*
Population	*8,296,693*
Official languages	*Spanish*
Total Area sq km	*112,492*
GDP (PPP)/per capita $	*3830*
Expenditure on health, % GDP	*7.3*
Life expectancy at birth m/f	*67/73 years*
People living with HIV/AIDS	*39,000*

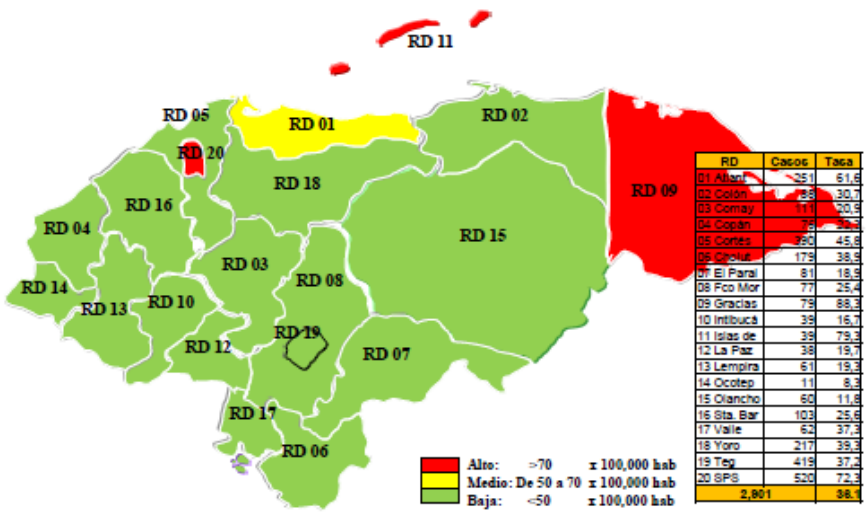
Honduras has incorporated the vaccine Bacille Calmette-Guerin (BCG) in the National Vaccination Program since 1977 in 298 districts in the country, with an increasing coverage range from 25% (1980), 55% (1985), 97%(1995), to 99% in 2008. The BCG vaccination is of obligatory use in newborns in the National Health System; yet, the protection of the vaccine is limited to meningitis in childhood, yet seems not to be protective against pulmonary TB[8]. Therefore, a novel vaccine is a priority for TB control and challenges the goal to reduce the global incidence of active tuberculosis.

Figure1. Estimated tuberculosis incidence rates by country, 2012[5]. Global tuberculosis report 2013. WHO 2013.



22 TB high burden countries: Afghanistan, Bangladesh, Brazil, Cambodia, China, the Democratic Republic of the Congo, Ethiopia, India, Indonesia, Kenya, Mozambique, Myanmar, Nigeria, Pakistan, The Philippines, the Russian Federation, South Africa, Thailand, Uganda, the United Republic of Tanzania, Vietnam and Zimbabwe. Combined, India and China account for an estimated 35% of the world's cases of TB.

Figure 2. Geographical distribution of tuberculosis in Honduras, all forms at intermediate level in 20 Health Regions. 2010.



RD	Casos	Tasa
01 Aflant	251	51,6
02 Colón	83	30,7
03 Comay	111	20,9
04 Copán	79	23,3
05 Cortés	990	45,8
06 Choluteca	179	38,9
07 El Paraí	81	18,9
08 Fco Mor	77	25,4
09 Gracias	79	88,3
10 Intibucá	39	16,7
11 Islas de	39	79,3
12 La Paz	38	19,7
13 Lempira	61	19,3
14 Ocotep	11	8,3
15 Olancho	60	11,8
16 Sta. Bar	103	25,6
17 Valle	62	37,3
18 Yoro	217	39,3
19 Teg	419	37,2
20 SPS	520	72,3
2,901		36,1

Secretaria de Salud, Programa Nacional de la Tuberculosis, Dr. Marvin Maldonado

2 PATHOGENESIS AND IMMUNITY OF TUBERCULOSIS, CHAPTER 2

Tuberculosis is a chronic infection acquired by the inhalation of infected particles from a patients with active TB. Small drops of 1-3 µ can reach the alveolar area containing approximately three bacilli per particle. However, it is not known what the minimal infectious dose of TB for humans is. Different numbers of bacilli may be required to establish infection, depending on the genetic background of the populations, regions, different *M.tb* strains, virulence, bacterial load, and the host immune response.
The exposition to *M.tb* and the *M.tb* directed host response display different clinical outcomes: i) asymptomatic: during infection-sterilization or latent TB infection (LTBI); ii) symptomatic phase during active disease manifestation. Most of the individuals are able to develop an effective cell mediated immune response to control the infectious stage, while 10% of exposed individuals do experience immune protection during their lifetime and may develop clinical disease either as primary infection or reactivation.
Once bacilli are ingested, alveolar macrophages engulf *M.tb,* they are deposited in the lung parenchyma, while macrophages others migrate through the blood stream to draining lymph nodes (DLN), where they encounter Dendritic cells (DC) for priming T-cells. Since *M.tb* is an intracellular bacterium that resides within the compartments of macrophages, presentation of specific mycobacterial antigens by Major Histocompatibility Complex (MHC) is needed to activate effector T lymphocytes, both CD4+ and CD8+ T-cells, and generate an inflammatory response orchestrated by cytokines and chemokines.

2.1 EARLY LINES OF DEFENSE

The nature of protective immune response against *M.tb* it is not completely understood. Most likely, it reflects an active interaction between the immune competences of the host the virulence of the pathogen.
The initial defense against TB are macrophages, they play role as target cells of *M.tb.* They represents also a first line defense and stimulate pro-inflammatory cytokines. The interaction between *M.tb* and macrophages is mediated by different sets of molecules, e.g. FcR, complement receptors (CR1, CR3, CR4), mannose receptors, surfactant protein receptors, CD14 and scavenger receptors[9]. After bacterias are engulfed, a chain of events starts, i.e. phagosome-lysosome fusion, generation of reactive oxygen intermediates, and generation of reactive nitrogen intermediates, particularly nitric oxide. Some of these events are actively inhibited by *M.tb* and represent immune evasion mechanisms. Simultaneously, T lymphocytes may be recruited to the macrophage and further stimulate it in order to inhibit *M.tb* growth or to kill mycobacteria.

Macrophages can also produce IL-12 to amplify this pathway. **Dendritic cells**- DCs are professional Antigen Presenting Cells (APCs) and are present in the lung tissue on the onset of inflammatory immune response as immature DC. Upon *M.tb* infection in the lung, DCs become activated through Toll like receptor signals. After antigen recognition, uptake and antigen internalization, DCs become mature and migrate to peripheral lymph nodes. During the migration and maturation process, DC may express surface markers, adhesion molecules, co-stimulatory molecules (CD80, CD86) accompanied of increased synthesis of MHC class I and II molecules. In the protective immune responses, DCs are believed to induce maturation of T cells towards a Th1 profile.

Neutrophils- PMNs are professional phagocytes with microbicidal effects, they produce chemokines (e.g. IP-10, MCP-1, MIP-1aB) and pro-inflammatory cytokines (e.g. TNF-α) in response to microbial pattern recognition receptors. They are believed to contribute in the first line of defense against TB[10]. This situation may also represent a double-edged sword, if neutrophils lead to exacerbated inflammation in the lung, evoking pathology and tissue damage.

Toll Like Receptors (TLR) - Their role is still controversial in immune response of TB. TLR-dependent activation of the NF-kB pathway is critical in infectious and inflammatory disease processes mediating cytokine activation signal transduction. This pathway is mediated via the adapter protein MyD88, which links to TLR signaling cascades, but also to IL-1B and IL-18 (IL-1BR, IL-18R). TLR signals promote production of microbicidal effectors molecules, such as ROI, RNI and microbicidal peptides (e.g. defensins). In a mouse model, TLR2 confers responsiveness to LAM, while heat killed *M.tb* elicit TLR-2 dependent responses, promoting the production of IL-2 and inducible nitric oxide synthase (iNOS). Therefore, distinct mycobacterial components can interact with different members of the TLR family.

Natural Killer cells (NK) - They are granular lymphocytes of the innate immune system that participate in allograft rejection and killing of transformed and viral-infected cells. In addition to their (perforin/granzyme- or Fas ligand-mediated) cytotoxic effector functions, NK cells produce pro-inflammatory cytokines, most prominently IFN-γ. Human NK cells are known to directly lyse *M.tb* infected monocytes and macrophages in vitro; but they also actively restrict mycobacterial growth in an apoptosis-dependent but Fas/FasL independent manner. Killing can be further enhanced by addition of IL-2, IL-12 and glutathione. NK cells are capable of mounting a vigorous response to *M.tb*, their exact function in vivo remains enigmatic[10].

2.2 ACQUIRED IMMUNITY: CELLULAR AND HUMORAL RESPONSES

The antigen specific, adaptive immune response execute several effectors functions via the activation of components of the innate immune system[11]. Early lines of defense and

adaptive immune response ensue, with the generation of antigen-specific effector cells that target the specific pathogen, as well as memory T-cells that can prevent re-infection - or control a latent TB infection.
Antigen specific immune responses can be differentiated into i) cell mediated mechanisms which include T-cell activation and effector mechanisms and ii) humoral immune responses consisting in B cell maturation and antibody production. In immune responses directed against *M.tb*, the role of B and T cell responses are not mutually exclusive. Once the bacilli activate dendritic cells, they will stimulate T lymphocytes, which in turn provide help to B cells and orchestrate adaptive immune response. T cells target and promote apoptotic killing of pathogen-infected cells either directly or through cytokine activation of neighboring immune cells. B cells may produce antibodies that neutralize invasion and target infectious agents for destruction, they also act as professional antigen presenting cells. T helper cells greatly impact the development and specialization of B cell responses and B cells conversely impact T-cell activation. Within this intricate relationship between both arms of immunity, cellular and humoral responses together determine the outcome of intracellular infection[12].

B cells forms a large part of bacterial induced immune responses to infection; they are detected in large numbers within granuloma lesions, yet the role of B-cells in immune protection is not clear[12].
Antibodies show properties to control infectious agents by preventing antigen entry and inhibiting replication, neutralizing toxins from pathogens and promoting antibody dependent cellular cytotoxicity, they lead to opsonization, trigger the complement cascade, Fcɣ receptor cross-linking and promote cytokine release. B cells function as Antigen Presenting Cells, regulate T cell differentiation and are able to induce T cell responses against certain intracellular microbes. Even though antibodies interact with extracellular pathogens to control infection*, M.tb* have both intracellular and extracellular stages in the course of TB infection that would allow antibodies to play a role during the entrance to the respiratory tract in the host or when *M.tb* escapes from the phago-lysosome in the macrophage. Yet researchers believe that *M.tb* usually resides within the vacuole. Thereafter antibody responses might allow production of effective immunoglobulin and passive immunization as potential defense in adaptive immune response. Intracellular pathogens like Clamydia species, Plasmodium, Cryptococcus neoformans and Schistosoma induce humoral and cellular mediated immune responses [13-15].
Experimental studies of *M.tb* are mainly based on murine models; pathologic progression of murine TB, or immune protection in murine models are ill-defined. This is also true for the biological role of antibody response against *M.tb* and *M.tb* (re) activation. The following examples show the relationship between cell mediated immunity (CMI) and

humoral immunity: in B cell deficient mice, deficiency of IgM μ-chain results in three to eight fold counts of viable bacilli a compared with healthy mice at 3-6 weeks post infection[16]. Other study showed that B cell deficient mice have exacerbated pathology and elevated numbers of pulmonary neutrophils [17] as wells as increased IL-10 production in lungs along with increased susceptibility to *M.tb* infection. Certain monoclonal antibodies demonstrated effects in limiting extra pulmonary *M.tb* dissemination. This included increased survival of infected animals and granuloma formation in case when BCG was coated with IgG2 and IgG3 anti heparin-binding haemagglutinin adhesion (HBHA) mAbs [18]; the same was found to be true for intranasal monoclonal IgA installation, i.e. anti α-crystalline in BALB/c [19]. Particularly *M.tb* antigens MPT83, Ag85A, Ag85B, HBHA and CFP-10/CFP-21 appear to induce protective antibodies [14].

Cellular immune response- Acquired immunity to *M.tb* relies on cell mediated immunity (CMI) that primarily target intracellular pathogens that reside in a vacuole within macrophages. Mycobacterial antigens are presented by and restricted to Major Histocompatibility Complex class II antigens. The increase of CD4+ T cells correlates to protection against TB, they are capable to induce effector cells to control mycobacterial growth and dissemination in the host. Essentially CD4 T cells produce Th1 cytokines like IFN-ɣ, IL-2 and TNF-α that play a critical role in controlling TB infection; particularly IFN-ɣ is important in controlling *M.tb* infection; it activates macrophages and induces the production of nitric oxide (NO) by nitric oxide synthase (NOS) which promotes killing of intracellular bacilli. CD4 T cells interact via CD40 and CD40L on macrophages and dendritic cells leading to enhanced antigen presentation.
CD8+ T cells participate in protection against TB associated with production of IFN-γ and subsequent activation of macrophage; IFN-γ promotes granzyme, perforine production. Antigen presentation to cytolytic T lymphocytes (CTLs) involve MHC class I molecules that can uptake the antigen from the cytosolic compartment, the endoplasmic reticulum. *M.tb* - infected macrophages undergo apoptosis and lead to the formation of vesicles containing mycobacterial antigens that can be processed by DCs presenting them in the context of MHC-II, MHC-I and CD1 [20-21].
'Unconventional T cells' are ill-defined in the context of protective immune responses of tuberculosis; these immune cells can also produce cytokines (IFN-γ) and produce cytotoxic activity, similar to CD8 CTLs. CD1 can present specific mycobacterial lipids and glycolipid peptides to T cells (in part TCR gamma/delta T-cells). CD1 molecules anchor short peptides and are structurally similar to MHC class I, yet the antigen binding groove is deeper and more hydrophobic than MHC I and II. Similarly γδ T cells are not only limited to cytokine production or to cytolytic effector functions, but they can efficiently

themselves present antigens and recognize phosphorylated ligands.

Interleukin 17-A

IL-17 is a potent pro inflammatory cytokine secreted by activated T lymphocytes and may contribute to granulopoiesis. IL-17 is secreted as a disulfide-linked homodimeric glycoprotein with a molecular weight of 30-35 Kda. IL-17 is overexpressed in a number of several pathological conditions including inflammatory airway disease, transplant rejection, multiple sclerosis or rheumatoid arthritis [22].

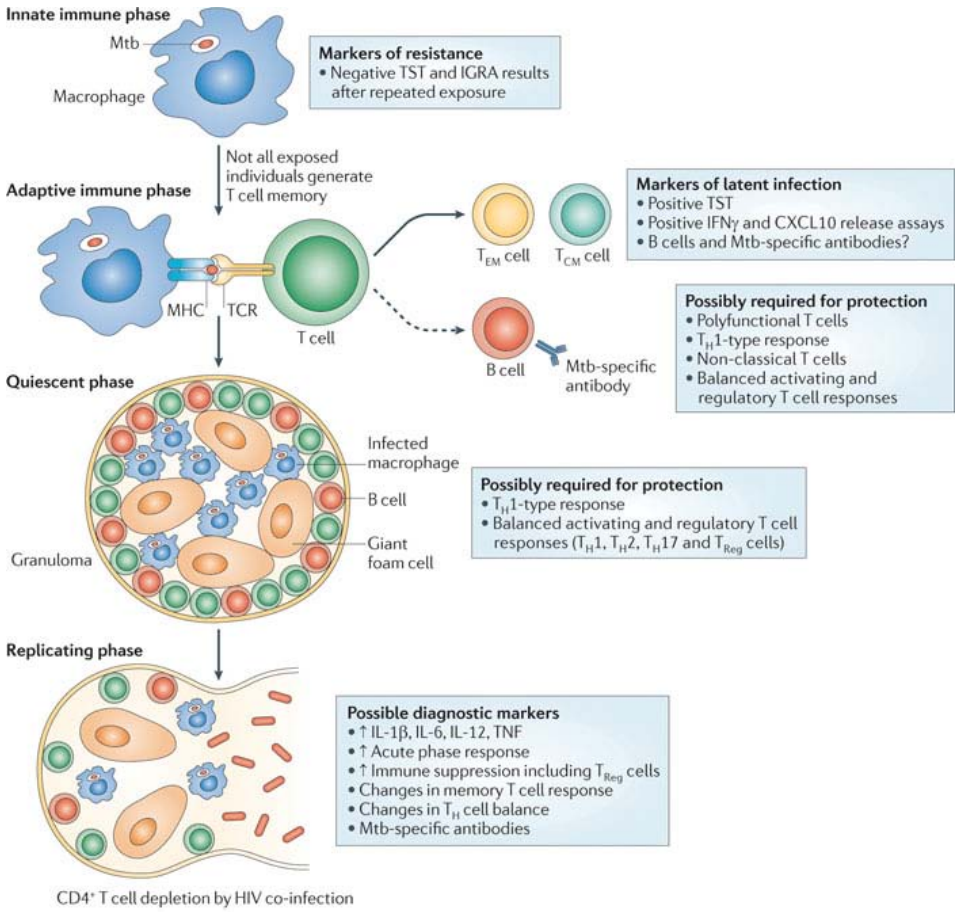
During *Mycobacterium bovis* BCG infection, IL-17 is expressed during both the early innate and the adaptive immune response, In BCG infected animals, γδ+T cells are the primary cellular source of IL-17, accounting for more than 60% of the IL-17 producing population. In turn, IL-17 contributes to acute neutrophil-mediated inflammation. In the absence of IL-17, a significantly lower number of neutrophils are recruited into the bronchoalveolar lavage fluid due to the decreased activity of neutrophils contributing to cytokine and chemokine production. In a human study comparing *M.tb* infected patients with healthy donors, the proportion of IL-17 producing γδ T cells decreased in patients with tuberculosis compared with healthy donors. Furthermore, the bacterial burden between T cell receptor γδ knockouts and wild type control mice did not differ significantly, yet granuloma structures were found to be less organized with a decreased composition of lymphocytes and monocytes after *M.tb* infection. Impaired granuloma formation is also observed in the lungs of IL-17 deficient mice infected with BCG, highlighting the importance of IL-17 in proper formation of tubercular granulomas [23].

2.3 QUIESCENT STATE AND GRANULOMA

One of the existing hallmarks in tuberculosis is the equilibrium of the host immune response and the mycobacteria provided by specific structures that contain the *M.tb* in a dormant state. It is currently debated if this containment can be maintained during life time and which mechanism tilt the balance to a non-effective immune response. The granuloma formation is an organized aggregate of immune cells as a consequence of cell activation to contain *M.tb* therefore preventing *M.tb* dissemination and active disease. It also involves remodeling of the lung parenchyma. This mechanism consists in the activation of a cellular recruitment network mediated by cytokines that provide cell migration, adhesion and localization; ICAM-1 (intracellular adhesion molecule) and TNF-α for instance play a key role in this process. The core of the granuloma shows caseation, necrosis, restricted nutrition factors and a lack of oxygen where the *M.tb* is able to persist with limited metabolic functions. The infiltration of cells around the granuloma core are concentric layers of macrophages, epithelial cells, multinucleated Langerhans cells,

lymphocytes and a fibrotic outer layer. David and Ramakrishna (2009) refute the classical formation and function of granulomas and have shown in a study of zebra fish embryos infected with *M. marinum* that granulomas accelerate and increase the mycobacteria expansion; however this model seems to be different to the human natural infection and biology.

Figure 3. Potential biomarkers of protection during tuberculosis infection. Different stages during pathogenesis help to differentiate cell compartments, pathways and peptides involved in replicating, non-replicating or quiescent mycobacteria



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2.4 CELL WALL STRUCTURE AND IMMUNOGENICITY

M.tb is different from other bacteria since it is surrounded by a complex cell envelope integrated by a cytoplasmic membrane and a cell wall; it represents a major virulence factor of *M.tb*, contributes to drug resistance and is responsible for *M.tb* survival under environmental stress. The cell wall consists in inner core of micolic acids, covalently attached lipids, carbohydrates and proteins intercepted in a matrix of peptidoglycans.

Long fatty acids chains compound micolic acids characterized for cyclopropane rings and double bounds, they link to peptidoglycan via an arabinogalactan polymer of arabinose and galactose subunits. The long branch, up to 90 carbon units forming micolic acids, account for 30-40% of the cell envelope mass; their depletion, lost or mutagenesis correlates with alterations in membrane structure, permeability and mycobacterial survival.

Several mycobacterial genes have been identified as virulence factors but biologically and clinically relevant key-genes and proteins for *M.tb* maintenance, persistence and survival of *M.tb* within macrophages, are still to be identified.

In this sense, proteomics and genomics have opened an opportunity for studying morphology, physiology and evolution of *M.tb*. The capacity of the *M.tb* genome, characterized by 4,411,529 base pairs (bp) and 3,924 open reading frames [24], provides gene products capable of being expressed during different stages of the TB infection eliciting cellular and humoral responses. The functionality of these genes shows *M.tb* lipid metabolism, cell-wall processing, virulence and antigenic variation, yet there still remains a 33% of hypothetical and unknown proteins to be elucidated [25-26]. The selection and understanding of TB antigens is a challenging perspective to define targeted cellular and humoral immune responses.

3 CORRELATES OF PROTECTION, CHAPTER 3

3.1 LIMITED EFFICACY OF BCG AND NEED OF EFFECTIVE VACCINE

The immune protection against tuberculosis aims to eliminate rapidly *M.tb*, including live mycobacterial-based vaccines as strategies. Despite several designs and attempts for an effective vaccine, all those efforts have been partially successful. Bacille Calmette and Guerin (BCG) is a live vaccine from an attenuated strain of *Mycobacterium bovis,* yet there is not protection against adult pulmonary TB and it limits only the systemic forms of TB in newborns. Since its introduction in 1921, different BCG vaccine strains variances exists due to genetics modifications occurred during repeated subcultures in different countries, these differences may be associated with the different protective efficacy against TB.

The particular BCG strain used worldwide depends of several factors such as logistics or cost, local production, or historical precedents. There is a range of global policies that regulates the use and age of BCG vaccination, doses and methodology for delivery. The BCG vaccination might affect the results in the Tuberculin skin test (TST) provoking cellular hyper-reactivity, this can be detrimental in immune-suppressed individuals. Furthermore, BCG vaccination is not useful any longer for the population already infected by *M.tb.* Despite nearly a century of use, BCG remains controversial with known variations of BCG sub strains, vaccine efficacy, policies and practices around the world [27].

3.2 BIOMARKERS OF PROTECTION AND NEW VACCINE CANDIDATES

There is a still a need to develop novel vaccines against TB that can be used in sensitized individuals, both due to exposition to environmental mycobacteria or previous BCG vaccination, or in (*M.tb*) latently infected subjects.

A biomarker of protection in the clinical context of vaccines and efficacy against disease is a “Feature that is objectively measured and evaluated as an indicator of a normal or pathological process of the response to an intervention” (Biomarkers definitions Working Groups, 2001). The lack of immune correlates of TB protection and the absence of biomarkers for vaccine candidates are challenges for vaccine development that need to be addressed.

Currently vaccine strategies include i) a pre-exposure vaccine for preventing disease and establish 'better' immune responses be improving the existing BCG i.e. recombinant rBCG, ii) a pre-exposure vaccine mounting a robust immune response before *M.tb* spreads in the host. iii) a post-exposure vaccine that prevents reactivation in a population with latent TB iv) a post-exposure vaccine that helps to prevent reactivation in high risk groups. Alternative strategies for TB vaccine also include antigens as potential boosters, currently in clinical trials are Ag85A, Ag85B, ESAT-6, CFP-10, TB10.4, Mtb72f, HBHA, which aim to prolong and strengthen the host immune response [28-29].

3.3 HETEROLOGOUS IMMUNITY AND CROSS REACTIVITY

Heterologous immunity is the term used to describe the phenomenon by which memory T cells that were generated during an earlier infection are reactivated in response to a second, unrelated infection or target. Each history of past infections may be able to shape the memory T cell pool. Private T cell receptor specificities of these preexisting memory T cell population influence both disease severity and outcome of subsequent unrelated infections [30-31].

The nature of T cell recognition is degenerate, a single T cell can often recognize more than a single epitope, one calculation suggesting as many as 10^6 peptide-MHC combinations [32]. CD8 T cells have been shown to be cross-reactive between two different epitopes from the same (viral) protein [33-34], between two proteins within the same virus [33, 35], between similar proteins of closely related viral pathogen [36-38], and between different proteins of unrelated viruses [39-40]. These cross-reactive epitopes may or may not have significant amino acid homology. Cross-reactive epitopes have also been shown between proteins of viruses and intracellular bacteria [41]. The mobilization of cross-reactive memory cells into a primary immune response can alter protective immunity, immunopathology, and the immune-dominance of subsequent T cell responses [36].

Welsh and Selin have broadly studied T-cell crossreactivity in infectious diseases suggesting that heterologous immunity can disrupt T cell memory pools, change pattern of T cell immune-dominance, lead to the selection of viral epitope-scape variants, alter the pathogenesis of viral infections, or contribute to variations in infectious diseases due to

the private specificity of T cell repertoires within individuals [42]. Epitope prediction and homology between sequences may allow identification of particular components for new vaccines and pathogenesis of tuberculosis.

4 ABOUT THIS THESIS

4.1 GENERAL AIM

The aim of this project is to better understand immune responses against *Mycobacterium tuberculosis* by defining *M.tb* specific immune responses in individuals with active and latent *M.tb* infection in Honduras using cell-based immune assays and a panel of specific *M.tb*. target antigens

4.2 SPECIFIC OBJECTIVES

- ☑ To identify the immunological profile in individuals with active and latent tuberculosis using different *M.tb* targets.
- ☑ Testing of novel *M.tb* targets useful for diagnosis and potential vaccine candidates.
- ☑ Implementation of the Quantiferon test and introduction/application of the Whole blood assay (WBA) detecting *M.tb* antigen –directed immune responses (screening).
- ☑ To characterize TB10.4 recognition in human and non-human primates (NHP) and to determine mimicry epitopes from environmental antigens in order to study cellular immune responses in TB and the role of repetitive antigen exposure.

4.3 SUMMARY OF THE PROJECT

Our research interest has being focused on *ex vivo* testing of potential tuberculosis vaccine candidates and *M.tb* antigens expressed at different stages of human TB, i.e. latent vs. clinically active TB. Individuals with latent or active TB were longitudinally monitored. The use of a broader panel of *M.tb* and MOTT (Mycobacterium other than tuberculosis) -

associated antigens may aid to dissect the precise targets of CD4- and CD8-mediated immune responses in *M.tb* patients. Thus, a broad array of *M.tb* antigens may be helpful to dissect immune responses to i) replicating bacteria, ii) 'quiescent' bacteria, ii) antigens able to differentiate between *M.tb*, MOTT and BCG (e.g. CFP10, ESAT-6). Of particular interest were *M.tb*-antigens expressed by non-replicating mycobacteria (persistence) and with clinical latent tuberculosis (since there is a need for a fast diagnosis of latent TB). Changes in both the magnitude of the response to some antigens and the nature of the immune response may allow us to better define the status of the TB infection.

In this study, we selected a well-defined population with newly diagnosed (TB positive cases and) a non TB population (health care workers and people with other respiratory diseases) which allowed us to determine biologically relevant information regarding to the immune response associated with the different status of TB infection. The project included the implementation of immunological based tests: Quantiferon Assay and WBA-Whole Blood Assay, Microarray Technology) with the antigens ESAT-6 (differentiation between *M.tb* vs. BCG and MOTT), Ag85A/B and TB10.4 (activity). IFN-γ was determined in the supernatants. Data was obtained and reviewed in association with clinical findings, results of the acid-fast staining, and isolation of viable bacteria. In a second set, the 'classical *M.tb* antigens (ESAT-6, Ag85A/B, TB10.4) were supplemented with an array of 13 *M.tb* peptide candidates and a set of 8 *M.tb* proteins to evaluate between replicating and non-replicating bacteria. Heparin-blood was ficolled and isolated PBMCs stored in liquid nitrogen to allow for the replication of experiments to further dissect cellular immune responses using intracellular cytokine staining (ICS).

In order to get specific immune response patterns, the population has been subdivided according to TB and HIV status. Data were obtained and correlated with the traditional TB diagnosis tests (acid fast staining and Löwenstein Jensen culture)

Study project summary

	M.tb Antigen testing	*Methodology*	*Parameters*	*Study Population*
Study I	***M.tb 15 mers long peptides n=13***			***1.1 TB+HIV- Culture & AFS positive***
	**** M.tb protein antigens n=8***	***Whole Blood Assay Quantiferon Assay ELISA Mycobacteriology***	***IFN-γ IL-17***	***1.2 TB-HIV- Control, other respiratory diseases***
	**** Classical M.tb peptides (n=3) & proteins (n=5)***			***1.3 TB-HIV- Previous TB and exposed Health care workers***
Study II	***1. TB10.4 Epitope Mapping. P1-P21***	***Whole Blood Assay Microarray Technology Luminex***	***IFN-γ B cell reactivity Multiple cytokine & chemokines***	***1.1 TB+,TB- subjects, NHP***
	2. Mimicry Peptide and Cross reactivity. M1-M8	***ELISA***		***2.1 TB+,TB- subjects***

4.3 MATERIALS AND METHODS

WBA Whole Blood Assay, WBA

We set up a T cell based assay (7 days) using diluted whole blood and specific TB antigens in order to measure T cell responses, cytokine production and cellular proliferation. Central memory T cell responses critical for long term protection are usually gauged in this assay. The cell cultures were incubated for 7 days at 37°C/CO_2 in duplicate wells; supplemented with RPMI/penicillin/streptomycin media in order to get supernatant containing lymphoblast and cytokine production in response to *M.tb* antigens. Results are provided by quantitative measurement of cytokines by ELISA.

Recombinant / Synthetic *Mycobacterium tuberculosis* antigens

TB bacterial antigens were provided by peptides, 15-mers long; Peptide pools were synthesized by JPT Peptide Technologies, Berlin, Germany at a final concentration of 1mg/ml. Recombinant proteins were used at 5mg/ml. A mixture of Staphylococcal Enterotoxin A (SEA) and B (SEB), (SEA/SEB; 10ng/ml) (Sigma Aldrich, USA) and Phytohemagglutinin (PHA; 5µg/ml) both were used as a positive control and RPMI medium (RPMI 1640 W/Glutamax supplemented with 1% Hepes and 0.5% Penicillin-Streptomycin)

without any stimulation as negative control. Peptide pools and proteins are described in the table below.

Gene locus	RefSeq	Antigen name
		Peptides
Rv0447c	NP854118 (Pool 1)	Probable cyclopropane Fatty acyl phospholipid synthase. ufaA1. (*M.bovis*)
Rv2940c	YP_976584 (Pool 2)	Mycocerosic acid synthase. mas. (M.bovis)
Rv3347c	YP_177963 (Pool 3)	PPE family proteins. PPE55. (*M.tb)*
Rv2453c	CAA16030 (Pool 4)	Probable molybdopterin-guanine dinucleotide biosynthesis Protein A. mobA. (*M.tb*)
Rv1886c	CAB10044 (Pool 5)	Antigen 85B. fbpB.
Rv1690	CAB10947 (Pool 6)	Probable lipoprotein. lprJ. (*M.tb*)
Rv3019c	CAA16104 (Pool 7)	ESAT-6 like protein. esxR. (*M.tb*)
Rv2957	CAB05419 (Pool 8)	PGL/p-HBAD biosynthesis glycosiltransferase. MT3031. (*M.tb*)
Rv1085c	CAA17201 (Pool 9)	UPF0073 membrane protein. MT1117. (*M.tb*)
Rv0066c	CAA16247 (Pool 10)	Isocitrate dehydrogenase, NADP-dependent- icd2. (*M.tb*)
Rv2958c	CAB05418 (Pool 11)	PGL/p-HBAD biosynthesis glycosiltransferase. MT3034. (*M.tb*)
Rv2962c	CAB05415 (Pool 12)	PGL/p-HBAD biosynthesis rhamnosyl-transferase. MT3038. (*M.tb)*
Rv0288	CAA17363	TB10.4 (pep pool). Low molecular weight protein antigen 7. esxH. ESAT-6 like protein. (*M.tb*)
Rv3804c/1886	CAA17868/CAB10044	Ag85A/Ag85B. Fibronectin binding protein -peptide pool- fbpA/fbpB
Rv3875/3874	CAA56099/CAA17966	ESAT-6/CFP-10. Peptide complex EsxA/EsxB
		Proteins
Rv3804c	CAA17868	Ag85A. Secreted antigen 85A. Mycolyl transferase 85A. fbpA. Ag85 complex. (*M.tb*)
Rv1886c	CAB10044	Ag85B. Secreted antigen 85B. Mycolyl transferase 85B. fbpB. Ag85 complex. (*M.tb*)
Rv3875	CAA56099	ESAT-6. 6 KDa early secretory antigenic target. esxA. (*M.tb)*
Rv3874	CAA17966	CFP-10. 10 KDa culture filtrate antigen. esxB. (*M.tb*)
Rv0754	CAE55319	PE_PGR11. PE-PGRS family protein. (*M.tb*)
Rv0978c	CAE55343	PE_PGR17. PE-PGRS family protein. (*M.tb)*
Rv1917c	CAE55440	PPE34. PPE family protein. (*M.tb*)
Rv0288	CAA17363	TB10.4. Low molecular weight protein. (*M.tb*)

IGRA- Interferon Gamma Release Assay, Quantiferon TB Gold in Tube

We performed the assay in test tubes including a negative control (Nil tube), positive control (mitogen tube) and a tube with TB antigens (ESAT-6, CFP-10, TB7.7) incubating all at 37°C 16-24 hours; after the incubation period, tubes were centrifuged and plasma collected to measure the amount of IFN-γ by the Enzyme Linked Immunosorbent Assay. Interpretation was done using specific software including an internal quality control and a standard curve. A positive result is considered if IFN-γ in the antigen tube is clearly superior to the IFN-γ Nil antigen in UI/ml (more than 2 standard deviations). The mitogen tube is used to determine the positive results of each sample; a low stimulation of mitogen (<0.5UI/ml) indicate an

undetermined result when the blood sample presents a negative reaction. Individuals with Nil values greater than 8UI/ml are classed as “indetermined” because a 25% higher response to the TB antigens may be outside the assay measurement range; this may occur due to excessive levels of IFN-γ or presence of heterophile antibodies or deviation of the procedure. A positive result for Quantiferon is when: TB antigen – Nil >/ 0.35 UI/ml , TB antigen – Nil >/ 25% of Nil UI/ml value , Nil </ 8UI/ml. The result is negative when the conditions mentioned above are not fulfilled.

Microarray Peptide Technology

Testing immune reactivity of TB antigens was performed by using a Microarray Peptide, a serum-antibody based test. The generation of binding patterns helps to identify antigenic epitopes and characterize vaccine targets, it is also valuable in monitoring immune responses in vaccine trials. The recognition of the antigen often depends of its conformation identifying immunoglobulin’s secreted by primed B cells [43].

In our study, the antigen array is a glass slide coated with several thousand of peptides from *M.tb* antigens. These peptides are attached on a solid surface detecting the presence of equal number of antibody specificities. The adaptive immunity generates specific antigen receptor with a high level of affinity in response to a stimulus. The aim of antibody profiling is the determination of immunoglobulin’s secreted by primed B cells with target epitopes estimating effector functions triggered by the binding events.

The serum reactivity of *M.tb* was studied using a glass array of 15 mers with overlapped peptides derived from *M.tb* protein. Serum was incubated with for 16 hours, after several washing steps, a secondary antibody Cy5 was added which is conjugated to a fluorescent probe followed by washing steps and drying the slide. The correspondent activity is measured by fluorescence intensity using a wave length at 635nm in a digital scanner GenePix Pro 5.1 software (Axon Instruments) which provides raw data reflecting the antibody immune responses of each individual to defined peptide vaccines.

Luminex, Bioplex Assay

This system of technology is based on the principle of flow cytometry to stream beads in single file past a pair of lasers. The Luminex immune-detection assay (Milliplex® MAP, Stockholm, Sweden) aims to analyze a large number of cytokines and chemokines based on color codes beads, called microspheres, read on a specialized analyzer. Within the Luminex compact analyzer, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay.

Each microsphere has a unique spectral signature using different intensities of dyes reaching up to hundred tests in a single reaction. The microspheres are coated with specific capture antibodies in order to react with the analyte in our stimulated cell culture supernatant, together with standards and quality controls.

The stimulated supernatant was tested in order to determine multi-analytes in a low amount of sample. After incubation and washing steps, biotinylated detection antibody is added and followed by a conjugate of Streptavidin-Phycoerythrin. The beads are washed and re-suspended in a sheat fluid to prior analysis in Luminex instruments (Luminex 200™) using two different lasers of light source exiting the internal beads and PE conjugate or any reported dye captured during the assay measuring Median Fluorescent Intensity (MFI) and calculating cytokine and chemokines concentrations (pg/ml) with curve-fitting method.

IFN-γ and IL-17 ELISA

During our study we performed the IFN-γ and IL-17A; ELISA (Eli-pair DIACLONE, Biosite, Stockholm, Sweden). The sample, supernatants from stimulated immune cells, were tested by ELISA. The basic principle of ELISA is to use an enzyme to detect the covalently linked binding of antigen-antibody. The enzyme converts a colorless substrate (chromogen) to a colored product indicating the binding of antigen-antibody. In brief, Nunc-Immuno™ Maxisorp 96 well plates were coated with the specific unlabeled capture antibody overnight at 4°C. Next day, the plates were washed with PBS 0.05% tween 20 to remove unbound antibodies, then the plate was blocked with PBS 5% Bovine serum albumin. The supernatants were collected from the Whole Blood Assay, they were thawed and diluted with PBS containing 1% BSA (1:1.47 in IFN-γ and 1:1.91 for IL-17). For IFN-γ, the reconstituted recombinant standard and samples were co-incubated with biotinylated

detection antibody for 2 hours at room temperature (RT). For IL-17, samples were incubated with coated antibody during two hours, washed three times and incubated with the detection antibody for 1 hour at room temperature. In both assays, after 3 washing steps, the plates were further incubated at RT with HRP-Streptavidin for 20 minutes. After washing step, TMB was added into the wells and plates were further incubated for 10-15 minutes in the dark. The reaction was stopped by adding 1M H_2SO_4 each well and the plates were read for absorbance at 450nm. A linear standard curve was drawn in order to obtain the unknown cytokine concentration where O.D. values were extrapolated with the given standard curve. The values were multiplied by their corresponding dilution factor and the concentration was expressed in pg/ml. The detection range for INF-γ was 400-7 pg/ml and for IL-17 100-3.1 pg/ml.

Cell Separation and Cryopreservation

During the developing of the study we collect peripheral blood mononuclear cells (PBMC) in order to get access to more detailed information. We implement the use of Vacutainer cell preparation tubes (CPT) with sodium heparin for peripheral blood collection and further lymphocyte purification using a rapid centrifugation procedure and assessment of the cellular viability. The cell separation medium is comprised of polyester gel and a density gradient liquid.

Samples were mixed properly by inversion prior to centrifugation; tubes were centrifuged during 15 minutes at 2800rpm at room temperature. After this step, mononuclear cells and platelets will be in the layer just under the plasma layer; the cells are suspended into the plasma and the entire content is transferred to 50 ml tubes, pooling the 2-3 CPT tubes of each patient and centrifuged during 10 minutes at 1700rpm and the plasma is separated and frozen at -80C freezer. The cell pellet is re-suspended and rinsed with PBS 1ml and then approximately the same blood volume from start and centrifuge the tube for 10 minutes at 1500 rpm and discard the supernatant; the cells are suspended in R10 medium and taking a cell suspension of 100-200 µl for cell counting in a Neubauer Chamber. Further we record the total cell counting and viable cells. A final centrifugation step is performed 1250rpm during 10 minutes. The cell pellet were re-suspended and preserved with freezing medium 10% DMSO in RPMI-FCS20% into cryovials ($1x10^6$ cells/ml), kept in a freezing device (prefilled with isopropanol) at -80°C during 12-24 hours to further transfer to liquid nitrogen.

Löwenstein Jensen Culture

Bacteriology isolation, culture and identification of sputum samples were performed by using Löwenstein Jensen solid media and decontamination-Petroff methodology.
The nutrients provided by monopotassium phosphate, magnesium sulphate, magnesium citrate, asparagine, potato flour and malachite green as inhibitor of accompanied flora; as well as an aggregate of glycerin to stimulate the growth of *M.tb*.
The inoculums is performed in duplicate above the surface of the media, incubating them up to eight weeks observing and reporting weekly the colonial morphology, pigments producing or contaminations. Positive cultures are further identified with biochemical test of catalase production, niacin and reduction of nitrates to nitrites.

4.5 SUMMARY OF RESULTS AND DISCUSSION

STUDY I

Increased IFN-γ production to M.tb antigens in blood from health care workers exposed to M.tb as compared to TB patients

We analyzed and identified in blood from 148 individuals a significant difference in IFN-γ production in response to peptides Rv2958c, Rv2962c, Rv3347c, Rv3804c, and Rv1886c (protein): between groups 1 (TB+) and 3 (health care workers); the antigens Rv2958c and Rv2962c were differentially recognized between group 2 (non-TB pulmonary diseases) and 3 (health care workers). Immune cells from individuals in group 2 (TB-other respiratory diseases) and group 3 (exposed to TB, no clinical TB uninfected) produced significantly higher levels of IFN-γ in response to stimulation with Rv1886c (Ag85B) Rv2958c (glycosyl-transferase) and Rv2962c (pHBAD biosynthesis rhamnosyl-transferase). The recombinant protein antigens Rv3804c (Ag85A) and Rv3347c (PPE family member) induced the strongest IFN-γ production in blood from *M.tb* exposed individuals as compared to TB + individuals.

A different M.tb target recognition pattern defined by IL-17 production

We selected therefore 14 *M.tb* antigens for further analysis and tested IL-17 production in response to the antigens Rv0447, Rv1886c, Rv3019c, Rv2957, Rv2958c, Rv2962c (peptides) and the recombinant proteins Rv3804c, Rv1886c, Rv3874, Rv3875, Rv0288, Rv0754, Rv0978a and Rv1917. We were able to demonstrate significantly different IL-17 production in response to Rv1886c (Ag85B) in individuals from group 2 (non TB, other respiratory disease) and 3 (health care workers, exposed to TB). Immune cells from individuals in group 2 and group 3 produced more IL-17 in response to Rv0978c (PPE family member), Rv0288 (TB10.4) and Rv1917c (PPE family member). The highest IL-17 production was identified in blood from exposed individuals directed against the antigens listed above.

Analysis between QFT-GIT and bacteriology

We analyzed the cytokine responses in blood from individuals stratified by the QFT-GIT based on the acid fast stain result, culture positivity and previous history of TB). Immune cells from individuals with AFS + *M.tb* + culture showed decreased IFN-γ responses to the positive (SEA/SEB) controls. ESAT-6 and CFP-10 immune reactivity was significantly higher in blood from individuals with AFS- and negative culture results– yet with a positive QFT-GIT for IFN-γ production (and for IL-17 production in response to Rv3874, CFP10). Significant differences concerning cytokine response patterns could be identified in blood from patients with TB (AFS+, QFTGIT+ and culture+) versus blood from health care workers in response to Rv3804c (Ag85A, IFN-γ $p = 0.009$ as well as for IL-17, $p = 0.019$) and Rv2962 (pHBAD, rhamnosyl-transferase, $p = 0.042$ for IL-17 production).

Discussion

Anti-*M.tb* directed immune responses may be associated with immune-protection, yet also with immune-pathology, as shown for IL-17-driven cellular immune responses. IL-17 – associated immune responses can be protective or harmful in TB and may lead to lung tissue damage along with massive inflammation and influx of neutrophils. We were able to show that IFN-γ mediated responses showed strong cellular recognition of Rv1886c (Ag85B), Rv3804c (Ag85A), Rv2958c, Rv2962c (enzymes associated with lipid-alteration) and Rv3347 (PPE family member) in blood from individuals who have been exposed to *M.tb*, yet are clinically healthy.

Of interest is the quite different cellular reactivity concerning *M.tb* target pattern recognitions if IFN-γ and IL-17 are analyzed. Ag85B (Rv1886c) showed a similar trend concerning T-cell recognition, both for IFN-γ and IL-17 production. Yet the antigen Rv0288 (TB10.4) and two PPE family members (Rv0978c, Rv1917c) exhibited only statistical differences in IL-17 responses, yet not concerning the capacity to induce IFN-γ production. Vice versa, the PPE family member Rv3347c, the enzymes Rv2958c (glycosyl-transferase) and Rv2962 (rhamnosyl-transferase) showed only differences in IFN-γ production between the populations (groups 1, TB + and 3, health care workers).

Several explanations may apply for the differential recognition patterns defined by IFN-γ and IL-17 production: Rv2958c and Rv2962 were tested as overlapping peptides; some peptides may be degraded and therefore not efficiently processed and presented to T-cells; the antigen processing and presentation of peptides may be different as compared to recombinant proteins. IL-17 production may require the intact protein structure and subsequent cellular processing and presentation. Other cellular mechanism may be important to initiate IL-17 production, i.e. the priming and activation of antigen presenting cells which will subsequently present the recombinant target protein to antigen-specific T-cells.

Of interest is the strong recognition, defined by IL-17 production, of TB10.4 (Rv0288c) and Ag85B (Rv1886c), two components of several TB vaccine candidates, in blood from healthy TB- exposed individuals. The strong TB10.4 recognition in blood from healthy individuals appears to be in contrast to the study of Sutherland and coworkers who reported a significant difference in IFN-γ production, yet not IL-17 production, by comparing cytokine responses in blood from TB + cases (West Africa) and a TST- control cohort [67]. Several reasons may account for these differences, i.e. a different exposure history of the test population to *M.tb* and/or environmental mycobacteria and environmental pathogens and subsequent expansion of IL-17+ producing immune cells. Future studies may therefore need to dissect the role of the cell source of IL-17 production in response to *M.tb* targets and require the stratification of immune response analysis based on the distribution of immune cells subsets (T-cells, NK, NKT, TCRγδ + T-cells) in the test samples.

STUDY II

Dominant TB10.4 directed recognition of AWQAQWNQAMEDLVR in BCG-primed non-human primates.

Blood from NHPs were tested for detailed TB10.4 epitope recognition after the boost with rAD35, which followed the prime (BCG1331 or AFRO-1). 3 NHPs were not immunized and served as controls to gauge TB10.4 directed immune responses. T-cell responses directed against a single peptide (TB10.4 $P14_{53-67}$ AWQAQWNQAMEDLVR) were identified by IFN-γ production in the BCG and rBCG group. We could not differentiate whether the dominant recognition of peptide $P14_{53-67}$ was due to the prime or associated with the rAd35 boost. The fact that T-cells from NHPs, who were not BCG/AFRO-1 primed, yet only received the rAd35 boost, recognized the peptide $P14_{53-67}$, suggests that the dominant response towards the epitope $P14_{53-67}$ was associated with the boost. T-cells from non-vaccinated animals did not react to TB10.4 peptides. PBMCs from 5/24 animals did not exhibit recognition to any of the peptides. PBMCs from 1/24 animals exhibited not only reactivity to peptide P14, yet also to an additional peptide P20. PBMCs from 3/3 animals who had not been vaccinated showed IFN-γ production after the *M.tb* challenge directed against the P14, 1/3 NHPs reacted to P1 and 1/3 to P6. In summary, BCG vaccination resulted in strong recognition of peptide P14 and this pattern did not appear to change after *M.tb* challenge.

Humoral and cellular TB10.4 epitope mapping in TB+ and TB-exposed individuals.

TB10.4 protein for immune-reactivity was tested using 21 synthetic overlapping peptides. In 23 HIV-negative subjects (TB+, n=7 and TB-, n=16). Both TB+ and TB- negative groups show a similar pattern recognition pattern based on IFN-γ production, with a diverse recognition of the TB10.4 protein. PBMCs from 4/7 subjects in the TB+ group exhibited recognition of peptides (P) P6, P11, P14 and P20. The strongest IFN-γ production could be observed to P10, P2 and P11 (191, 110 and 109 pg/ml) respectively. Within the TB- group, recognition of peptides P1, P2 and P10 were shared among 7/16 individuals. T-cells from 4/16 subjects did not produce IFN-γ to any of the 21 TB10.4 candidate test peptides.

Next, we tested the TB10.4 protein for immune-reactivity using 21 synthetic overlapping peptides in 15 HIV-negative subjects (TB+, n=7 and TB-, n=8). The corresponding serums from the same individuals were tested for IgG recognition using peptide microarray technology. This pattern was different as compared to the recognition of peptides P 2, 3,

11, 12, 15 in the TB+, as well as in the TB- group defined by IFN- γ production. Anti - peptide AWQAQWNQAMEDLVR reactivity was detected in a single serum sample. Since the peptide TB10.4 AWQAQWNQAMEDLVR was strongly recognized in NHPs after BCG and *M.tb* challenge, as well as in blood from patients with TB, we tested closely related peptides from other species, labeled as M1-M8. Blood from 4/7 individuals of the TB+ group and 8/16 subjects from TB- group did not respond to any of the variant, yet to the wild-type peptide. The report shows T-cell responses within the TB positive group defined by IFN-γ production (between 8-60 pg/ml) with diverse recognition pattern including peptides from *S. cerevisiae* (M2) and to *R. lactaris* (M7). 50% of TB- individuals (TB-exposed individuals) showed a response to the 9mer peptide from TB10.4 (M8) followed by reactivity to a peptide from S. cerevisiae (M2) and to *A. fumigatus* (M4).

We tested additional cytokines (Th1/Th2) in blood from 4 TB+ patients and 4 TB-contacts who showed IFN-γ production towards AWQAQWNQAMEDLVR. Distinct cytokine production could be observed to the 9mer peptide from TB10.4 (M8) as well as to a peptide from *S. cerevisiae* (M2) and *A. fumigatus* (M4), defined by TNFα, IFN-γ and GM-CSF production. We could also observe IL-4 and IL-10 responses to variant peptides from *S. cerevisiae* (M2) and *A. fumigatus* (M4), as well as the 9mer peptide from TB10.4

Discussion

In this study we have investigated the cellular recognition pattern in response to targets from unrelated organisms that may skew the host immune response to TB10.4. Of interest is the consistent cellular immune response in PBMCs from NHPs to the TB10.4 peptide $P14_{53-67}$ (AWQAQWNQAMEDLVR) as a response to result of BCG vaccination or the Adenoviral-TB10.4 boost. We identified in this report the peptide $P11_{41-55}$ *SAWQGDTGITYQAWQ* to be frequently recognized in PBMCs from TB patients. PBMCs from *M.tb* – negative individuals recognized the peptide P_{25-19} (*MYNYPAMLGHAGDMA)* which has reported to be frequently recognized in patients with TB or individuals after BCG vaccination; an epitope which has reported to contain a dominant CD8+ T-cell epitope in murine *M.tb* infection that is able to induce cytotoxic T-cell responses. The differences in the cellular TB10.4 immune recognition patterns between NHPs and TB+ patients may be due to inherent genetic differences (e.g. MHC restriction, TCR repertoire), yet may also point to environmental factors; the 'clean' environment of *M.tb* infected (outbred) NHPs; in contrast to humans, NHPs may favor a focused anti-TB10.4 directed immune response.

PBMCS from individuals from Honduras frequently recognized the epitope SAWQGDTGITYQAWQ. Several environmental and / or genetic factors could account for these differences. Exposure to microorganisms may generate T cell cross-reactivity. Shared amino acid sequences of unrelated pathogens could be the target of memory T cells and shape the encounter with the nominal TB10.4 target antigen in course of *M.tb* exposure or BCG vaccination. Further studies may have to address whether cytokine production in PBMCs from patients with TB is indeed related to TCRs that recognize structurally similar peptide species from different bacterial or fungal species and whether this T-cell reactivity is biologically and clinically relevant in the development of anti-*M.tb* directed immune responses.

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